MORPHOLOGICAL CHANGES IN FROG PRONEPHRIC CELL SURFACES AFTER TRANSFORMATION BY HERPES VIRUS

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SUMMARY

A scanning electron-microscope study has established that there are major modifications in the surface membranes of malignant cells transformed from the pronephric kidney of the frog. Tumours were induced in vivo after exposure of embryos to Lucké Herpes virus. A pronephric cell line from normal embryos and 3 tumour cell lines derived from experimentally induced adenocarcinomas of the pronephros were observed. The normal embryonic kidney cells have their surfaces covered with long fingerlike microvilli throughout the cell cycle. In contrast, the surface of the pronephric tumour cells are mainly covered by multiple, ruffled or parallel straight microridges and short, stubby microvilli. These comparisons were made on cells subject to similar culture conditions. The topographic changes in the tumour cells are considered significant since the characteristic microridges and stubby villi were consistently found in all 3 pronephric tumour lines.

INTRODUCTION

When cells are transformed from the normal to the malignant state, changes in cell shape, size and surface morphology are attendant to functional modifications such as growth control, invasiveness, loss of contact inhibition and metabolic changes (Abercrombie & Ambrose, 1962; Stoker, 1971). Perturbations in the cell surface, particularly microvilli, have been correlated with spontaneously transformed cells in vitro (Boyde, Weiss & Vesely, 1972; Porter, Fonte & Weiss, 1974; Porter, Todaro & Fonte, 1973; Pugh-Humphreys & Sinclair, 1970). Similarly, cells transformed with an oncogenic virus (if not already possessing microvilli) may develop microvilli (Boyde et al. 1972; Vesely & Boyde, 1973; Perecko, Berezesby & Grimley, 1973) although surface blebbing (zeiosis), marginal filopodia, surface ruffling or folds often predominate instead (Porter et al. 1973; Hale, Winkelhake & Weber, 1975). Inoculation of the kidney primordia of frog embryos with oncogenic Herpes virus from infected pronephric cells has resulted in in vivo transformation of the pronephros (Tweedell & Wong, 1974). Several cell lines have been derived from the embryonic pronephros, a normal pronephric cell line (Wong & Tweedell, 1974) and 3 pronephric tumour cell lines (Tweedell, unpublished results). The characteristic surface morphology of normal pronephric cells in monolayer cultures was distinctly and consistently different from that observed in the tumour cells. The unique membrane changes observed in the viral-transformed tumour cells may relate to their function as neoplastic cells.
MATERIALS AND METHODS

The frog pronephric kidney cell line was derived from primary explants of pronephroi obtained from embryonic stages (Shumway, stage 24) of *Rana pipiens* indigenous to the Wisconsin-Minnesota area (Wong & Tweedell, 1974). This pronephric cell line, WMPA (Wisconsin-Minnesota pronephros, strain A) is predominately epithelioid with some fibroblast-like cells.

The frog pronephric kidney tumour cell lines were derived from experimentally induced renal carcinomas of the pronephroi from metamorphosing *R. pipiens*. Herpes virus derived from an adenocarcinoma of the adult were used to infect the WMPA cell line. A mitochondrial-Herpes virus fraction from the 3rd passage of this infected cell line (WMPA/V208) was used to inoculate *R. pipiens* embryos, stage 17, using the procedures described by Tweedell (1967). Inoculated embryos subsequently developed tumours of the pronephros and mesonephros (Tweedell & Wong, 1974).

One pronephric kidney tumour cell line (PNKT-3) was derived from primary explants of a pronephric tumour induced after embryo inoculation. A second pronephric kidney tumour (PRKT) line was obtained from cell dissociation of the same tumour. A third tumour line, PNKT-4 was started by primary explants of another induced pronephric carcinoma from the same series. All 3 cell lines are primarily epithelioid; more than 85% are polygonal and the rest are fibroblast-like cells.

Cell cultivation

Both normal and tumour pronephric cell lines were subcultured from flasks and plated at different densities on coverslips in Leighton tubes. Cells were grown in Leibovitz-15 culture medium modified for amphibian tonicity (Balls & Ruben, 1966) containing 10% foetal calf serum and incubated at 25 °C. Cultures were set to provide final cell densities ranging from less than 50% confluency to over 100% confluency. The culture medium was changed on the next day and all cells were fixed simultaneously at 48 h after plating. The final pH of the cultures was 7.2-7.3.

Preparation of cells for microscopy

Coverslips with attached cells were removed from culture and rinsed 3 times in amphibian Ringer's solution at room temperature (approx. 22 °C). The cells were then fixed for 45 min to 1 h in cacodylate-buffered 4% glutaraldehyde, rinsed 3 times in a cacodylate buffer and postfixed for 1 h in cacodylate-buffered osmium tetroxide. The fixation and rinse steps were carried out at room temperature. The cacodylate buffer solutions contained 0.1 M sodium cacodylate, 10⁻¹ M CaCl₂ and sufficient NaCl to bring the tonicity of the final solution to 500-550 mosmol. The cells were dehydrated through a graded acetone series (30-100%), rinsed 3 times in amyl acetate and critical-point dried in CO₂ (Anderson, 1951). They were then coated with thin layers of carbon and gold-palladium (20-25 nm) by vacuum evaporation and examined at 15 or 25 kV in a Cambridge Stereoscan 600 scanning electron microscope.

Fig. 1. A single epithelioid normal pronephric cell (WMPA) with abundant long microvilli and surface buds on the upper surface. Anchoring filopodia and lamellipodia are apparent around the cell periphery. × 2200.

Fig. 2. An enlargement of the cell surface seen in the preceding figure showing the abundance of long microvilli along with a few surface blebs and short villi. × 5800.

Fig. 3. A normal pronephric cell with extensive long microvilli over most of the cell surface. Cell extensions were sparsely covered and ended in lamellipodia. × 2200.

Fig. 4. Another epithelioid pronephric cell showing the predominance of long microvilli and some fusion at their bases. A few short villi are evident. × 5800.
RESULTS

Cultures of normal pronephric cells (WMPA) in the 89th to the 91st subculture were plated at different densities to yield monolayers of low, medium and high density. In low and medium density cultures both epithelioid and fibroblastoid cell types had their dorsal surfaces heavily covered with long, slender microvilli, often in disarray (Figs. 1–6). These long, fingerlike microvilli were usually single and of equal thickness (about 0.2 μm) from the base to the tip. They were prominent on the large pyramidal epithelioid cells (Figs. 1, 2). Their pattern of distribution was generally uniform over most of the cell. The interstitial surfaces between microvilli were flat and smooth. Patches of smooth surface were also found on the cell extensions which ended in rather well developed lamellipodia. The lateral edges of the cell had long, thread-like filopodia (retraction microfibrils) that were distinct from the long microvilli (Fig. 1).

Scattered round bulbular surface zyotic blebs (Porter, Prescott & Frye, 1973), either isolated or in linear or circular clusters, were often found near the cell centre (Fig. 2). Occasional short microvilli could be seen on the flat surface peripheral to the blebs (Fig. 3). Usually there was a mixture of many long microvilli and a few surface blebs. When surface blebbing was absent the cells were densely covered with long microvilli (Fig. 4). Sometimes 2–3 microvilli were fused at their bases into short vertical lobes that ended distally in long and thin microvilli. Lobes of basally fused villi were more evident in the rounded predivision cells. In these cells, the number of long microvilli increased and they were heavily concentrated over the central, mounded nuclear area. As cell monolayers became confluent, the long microvilli and surface blebs were concentrated toward the cell centre while the peripheral surfaces were smooth and free.

The spindleshaped fibroblast-like cells were more uniformly covered with similar-appearing long, fingerlike microvilli (Figs. 5, 6). Again, these microvilli were occasionally fused at their bases into short vertical lobes. The microvilli were often distributed along the entire length of the cell but were fewer along the cell extensions. One or both linear extensions of the fibroblast cell ended in plume-like lamellipodia. Surface blebs were also present singly or in clusters or rings above the nucleus (Fig. 6).

Fig. 5. A pair of fibroblastic normal pronephric cells (WMPA) also covered with long microvilli. The villi thin out along the cell extensions which terminate in lamellipodia. × 1200.

Fig. 6. An enlarged view of the left cell in Fig. 5 showing the relative length of the long, fingerlike microvilli. Several surface blebs have fused into a surface ruffle. × 5800.

Fig. 7. An isolated pronephric tumour cell (PNKT-4) with abundant stubby microvilli concentrated toward the cell extensions and some microridges over the upper, central cell surface. The distribution of stubby microvilli and microridges varied from cell to cell. × 2200.

Fig. 8. A mixture of convoluted microridges and surface blebs and relatively few microvilli are seen on this pronephric (PNKT-4) tumour cell from low-density culture. × 5800.
Transformed frog pronephric cells
**Pronephric adenocarcinoma cells**

Pronephric tumour cells (6 x 10⁶ cells/ml) were plated at densities of 30, 50, 60 and 95% to provide low, medium and high density monolayers. The PNKT-4 cell line was in the 47th to 50th subculture, the PRKT line in the 48th subculture and the PNKT-3, a stored line (12 °C) was in the 15th subculture. Cells observed in monolayers that ranged from less than 50% confluency to 85–90% confluency exhibited the same basic surface characteristics with only minor differences.

**PNKT-4 cells**

In the lower-density cultures of the pronephric renal carcinoma cells the epithelioid and fibroblastic cells had a high profile with broad terminal cell processes. The most distinctive and unique feature was the presence of short, irregular, wavy surface folds (microridges) and short, stubby microvilli (Figs. 7, 8). These vertical pleats and microprojections of the surface membrane were dispersed over the entire surface of the polylobular epithelioid cells (Fig. 7). In some cells either stubby microvilli or microridges predominate but they often interdigitated on areas of the same cell. In still other cases, microridges and short microvilli were found mixed on the same cell, but segregated into discrete patches (Figs. 7-9). Long threadlike attachment filaments (contractile filopodia) were seen around the periphery of both mounded and flattened cells (Fig. 8). Lamellipodia were poorly developed and appeared rather irregularly along the broader edges of the cell. Spherical surface blebs were also less obvious but were sometimes associated with microridges in the cell centre.

When the cells approached confluence they became flattened and the microridges, along with the stubby microvilli, were heavily distributed across the cell surface (Fig. 9). Dense patches of short microvilli could be found in some areas while in other regions the microridges covered most of the surface. The basal surface between the microridges and the marginal areas of the cell were unbroken and appeared smooth.

The vertical surface folds often developed into long, parallel microridges in cultures of completely confluent cells (Fig. 9). These cultures were composed of flattened, polygonal cells with tightly fused cell borders. The microvilli and microridges were more concentrated toward the cell centre above the nucleus as seen in the right portion of Fig. 9. They were generally absent in the peripheral areas. Prominent
microridges were found on the compact, round, predividing cells whose surface was deeply convoluted. Long, hairy microvilli were observed covering the cells only when they approached cell division.

**PRKT cells**

The surface conformation in this cell line was essentially identical to that seen in the PNKT-4 cell line (compare Figs. 8, 9 with 10). Cells with prominent microridges or stubby microvilli were often found adjacent to each other (Fig. 10). Closer examination of an area of predominate microridges reveals that stubby microvilli can be found either arising from the crest of the microridges or from the spaces between them (see Fig. 11). Sporadic surface blebs could also be observed in some areas of these cells (Fig. 10).

**PNKT-3 cells**

In this cell line, surface microridges and stubby microvilli again dominated the epithelial and fibroblastic cells; the microridges were shorter and more convoluted with less parallel orientation than in the previous cell lines. Stubby microvilli were very evident on the shorter surface plica. In most flattened epitheloid cells the dorsal surfaces were covered with dense, very short microvilli (Fig. 12). These stubby microvilli usually arose singly, but at times they were fused at their bases into short, vertical folds. Zeiosis was not commonly observed. As in the other tumour cell lines long microvilli were never observed on any of the cells except in those preparing for division.

**Discussion**

Most normal cells growing in vitro that have been examined by SEM show relatively few microvilli and blebs on their surfaces. Porter & Fonte (1973) reported that normal rat liver fibroblasts are free of microvilli, ruffles or blebs. Explants of normal hamster lung in vitro also showed few microvilli except during mitosis when the projections were prolific (Porter et al. 1973). The presence of microvilli is usually related to mitosis, when the cells round up and become covered with microvilli or in cells that are specialized for a certain task (Enlander, Scott & Tobey, 1973). Large numbers of microvilli, filopodia and lamellipodia are the normal surface components of monocytes in tissue culture, apparently reflecting their invasive, phagocytic activities (Parakkal, Pinto & Hanifin, 1974).

Many embryonic cells in culture have greatly reduced surface microvilli. Mouse embryo squamous endothelial cells (BALB) have few and short microvilli (Porter et al. 1973). The normal fibroblast embryonic Lewis rat cells (LWF) are flat and devoid of microprojections (Vesely & Boyde, 1973). Embryonic fibroblastic cells in culture do show variations during the cell cycle. Chick embryo fibroblasts have a smooth surface during G1 and S-phase but develop extensive microvilli during the G2 period that persists through mitosis. When the cells become density-inhibited during G1 their surface becomes smooth (Hale et al. 1975).
The normal frog pronephric cells differed, since they were covered with long, slender microvilli and bulbular blebs in cultures of both low and medium density. Only when the cells became confluent and contact inhibited was there a decrease in microvilli. In comparison, kidney cells of the baby hamster (BHK21/C13) and fibroblast-like baby mouse kidney cells (CBM 17) both had only a few short microvilli, but microvilli were observed to be quite long and numerous on polygonal epithelial cells of the latter line (Hodges & Muir, 1972). In comparison both fibroblastic and epithelial cells of the frog pronephric line were consistently covered with long microvilli and bulbular blebs that were found throughout the cell cycle.

Cells that have undergone spontaneous transformation usually have large numbers of microvilli on their surface when viewed with the scanning microscope. Cultured HeLa cells, Lanschutz ascites tumour cells and canine Madin kidney cells all possessed microvilli on 90% or more of their cell surfaces (Pugh-Humphrey & Sinclair, 1970). Numerous microvilli and microridges are found on the epithelial-like cells of mouse sarcoma (S180) cells (Boyde et al. 1972). Many dense microvilli are seen over the central nuclear area of the rat sarcoma 4337 and long microvilli are a feature of neuroblastoma cells (Porter & Fonte, 1973). Following in vitro transformation of the embryonic fibroblast Lewis rat cells (LW 13) the dorsal cell surfaces became very hairy with many finger-like microvilli (Vesely & Boyde, 1973). Epithelioid HeLa cells (Porter et al. 1974) and spontaneous transformants of mouse BALB/3T3 cells showed similar phenomena (Porter et al. 1973).

Specific modifications of the cell surface even appear to vary according to the stage of the cell cycle. In synchronized Chinese hamster ovarian cells (CHO) microvilli, blebs and ruffles occur in definite sequence as they pass through stages of the cell cycle. The microvilli persist throughout the cycle but are most dense at mitosis and in the G1 period (Porter et al. 1973). In comparison, Rubin & Everhart (1973) observed that the transformed CHO cells, when synchronized and plated at low densities retained the surface configuration of the G1 phase, i.e. microvilli and blebs over the central cell body, and they did not express surface changes as the cycle progressed. Intercellular contact was required to bring about the additional changes, marginal ruffling and predominate filopodia during the S- and G2 period.

After transformation by an oncogenic virus alterations in cell surface morphology often occur. These modifications can take the form of microvilli, ridges, ruffling or blebbing. When mouse embryo (BALB/3T3) cells were permanently transformed by the exogenous simian virus SV40, they developed long, 'hairy' microprojections (Perecko et al. 1973). After similar exposure of Lewis epithelioid rat sarcoma cells (LW13) to Rous sarcoma virus, the transformed cells (RSK4) became very 'high and hairy' with long multiple microvilli that showed some fusion at the bases (Vesely & Boyde, 1973). Porter et al. (1973) however found that several viral transformants of BALB/3T3 cells by SV40, murine sarcoma virus, and polyoma virus developed surface blebs, some ruffling but few or no microvilli.

Transformed Lewis rat cells (LW13) already possessing microvilli and subsequently infected with Rous sarcoma virus, maintained the microvilli in heavy concentrations. Synchronized chick embryo cells after transformation by Rous sarcoma virus had
a surface that resembled the mitotic cell morphology. Typically, they presented a rounded formation and became covered with surface blebs and folds (Hale et al. 1975). Other cells lacking microvilli normally, such as quail fibroblasts, seem to acquire them after transformation (Boyde et al. 1972).

While it is tempting to correlate the presence of multiple microvilli with cell transformation (Porter & Fonte, 1973) it is obvious that exceptions occur, particularly following transformation by an oncogenic virus. The present pronephric cell system represents such an exception. The consistency of the differences observed in the surface membrane formations under identical culture conditions on the frog pronephric cells before and after transformation appears significant. Presumably they reflect changes in the embryonic pronephric cell that result from the in vivo transformation of the normal pronephric cells by the Lucke Herpes virus. Each of the 3 tumour cell lines, derived from tumours produced in the differentiating pronephros, developed the same form and pattern. A special adaptation appeared to be the presence of long, straight or ruffled microridges in the transformed pronephric cells that persisted throughout the cell cycle. A second change was the appearance of short, stubby microvilli in place of long microvilli. The parallel arrays of microridges supporting short, stubby microvilli developed in the tumour cells are very similar to parallel undulations possessing short microvilli that are observed along the dorsal lip of the blastopore during gastrulation of the frog embryo (Brick, Schaeffer, Schaeffer & Genaro, 1974). It is interesting to speculate that these unique changes in the surface of pronephric tumour cells also represent adaptations that permit unrestrained proliferation of the transformed cell in vivo.

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